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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/832,865	04/12/2001	Hua Yu	USF-T142X	1377

23567 7590 03/25/2005

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CHANTILLY, VA 20153

EXAMINER	
GAMBEL, PHILLIP	

ART UNIT	PAPER NUMBER
1644	

DATE MAILED: 03/25/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

PTO-90C (Rev. 10/03)

Office Action Summary	Application No. 09/832,865	Applicant(s) YU, HUA	
	Examiner Phillip Gambel	Art Unit 1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *09 January 2005*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-9* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-9* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Applicant's amendment, filed 10/12/04, has been entered.
Claim 1 has been amended.
Claims 10-15 have been canceled.

Claims 1-9 are pending.

Claims 1-9 as they read on the elected species of tumor cells and the combination of GM-CSF and IL-12

2. Upon reconsideration of applicant's amended claims, filed 12/12/04, in conjunction with the arguments that the claimed subject matter is drawn to cells of the method which are engineered to express soluble CD40 and not membrane bound CD40, the previous rejection under 35 USC 102(e) as being anticipated by Hoo et al. (UY.S. Patent No. 6,482,407),has been withdrawn.

3. Claim 4 is rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 4 is indefinite in that it lacks proper antecedent basis for "said tumor cells".

Applicant is reminded that the amendment must point to a basis in the specification so as not to add any new matter. See MPEP 714.02 and 2163.06

4. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office Action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

5. Claims 1-9 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Dranoff et al. (U.S. Patent No. 5,904,920) (1449; #B) in view of Heath et al. (WO 94/04570) (1449; #H) and Levitsky et al. (J. Immunol. 156: 3858-3865 (1996).

Dranoff et al. teach methods of regulating immune response utilizing cytokines and antigens, including the transfection and administration of tumor cells transfected with cytokines such as GM-CSF and IL-12 (e.g., see Summary of the Invention; Detailed Description of the Invention, including column 6, paragraph 2; column 8, paragraphs 4-6), wherein said tumor cells are rendered proliferation incompetent, including the use of irradiation (e.g. column 8, paragraph 2) (see entire document, including Detailed Description of the Invention).

Dranoff et al. differs from the claimed methods by not including soluble CD40 in the transfection of tumor cells to increase immune reponses and by not teaching B cell lymphomas as a target for treatment with CD40 as well as GM-CSF and IL-12.

Levitsky et al. teach GM-CSF transduced tumor cell-based vaccinations for B cell lymphomas (see entire document, including Abstract). Levitsky et al. teach the effectiveness of the paracrine production of cytokines by tumor cells in the generation of effective immune responses (see Discussion).

Heath et al. teach that CD40$^+$ malignancies such as B cell lymphomas are sensitive to inhibition via soluble CD40 (see entire document, including page 3, paragraph 3 and page 21, paragraph 1). Heath et al. further teach the recombinant production of soluble CD40, including introducing CD40 by an expression vector into host cells (see Soluble CD40 Molecules on pages 6-13)

Therefore, it would have obvious to a person of ordinary skill in the art at the time the invention was made to apply the teachings of Heath et al. to those of Dranoff et al. by including the transfection of soluble CD40 into tumor cells, particularly CD40$^+$ malignancies such as B cell lymphomas, to increase the effectiveness of tumor-based vaccination regimens, by taking advantage of the paracrine functions of GM-CSF and IL-12 in generating appropriate anti-tumor responses, and by taking advantage of the sensitivity of said CD40$^+$ malignancies to soluble CD40.

Accordingly, one of ordinary skill in the art at the time the invention was made would have been motivated to transfect CD40$^+$ tumor cells, such as B cell lymphoma with those cytokines (e.g. GM-CSF and IL-12) that stimulate the appropriate anti-tumor responses along with the appropriate agent that specifically inhibits CD40:CD40 ligand interactions and signaling in said tumor cells to increase the efficacy of tumor cell vaccinations by addressing different targets by increasing host immune responses while decreasing stimulatory signals to said CD40$^+$ malignancies, wherein said combination regimens were consistent with practices known at the time the invention was made, as evidenced by Dranoff et al.

From the teachings of the references, it was apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

6. No claim allowed.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Phillip Gambel whose telephone number is (571) 272-0844. The examiner can normally be reached Monday through Thursday from 7:30 am to 6:00 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on (571) 272-0841.

The fax number for the organization where this application or proceeding is assigned is 571-273-8300

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Phillip Gambel, PhD.
Primary Examiner
Technology Center 1600
March 21, 2005

Phillip Gambel

Notice of References Cited	Application/Control No. 09/832,865	Applicant(s)/Patent Under Reexamination YU, HUA	
	Examiner Phillip Gambel	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
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FOREIGN PATENT DOCUMENTS

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	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	LEVITSKY ET AL. J. IMMUNOL. 156: 3858-386~~4~~ (1996)
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	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Immunization with Granulocyte-Macrophage Colony-Stimulating Factor-Transduced, but Not B7-1-Transduced, Lymphoma Cells Primes Idiotype-Specific T Cells and Generates Potent Systemic Antitumor Immunity[1]

Hyam I. Levitsky,[2]* Jami Montgomery,* Mojgan Ahmadzadeh,* Kevin Staveley-O'Carroll,† Frank Guarnieri,‡ Dan L. Longo,§ and Larry W. Kwak§

Recently, genetically modified tumor cell vaccines have been described for nonhematopoietic cancers in which the relevant Ags are unknown. Several of these cell-based vaccine strategies have been shown to induce T cell-mediated systemic antitumor immunity, either by enhancing the processing and presentation of tumor Ags by host APCs or by facilitating effective Ag presentation by the tumor vaccine itself. These strategies were compared in a model B cell lymphoma, a tumor derived from APCs, which have the inherent capacity to activate Ag-specific T cells. Eradication of pre-established systemic lymphoma was achieved following immunization with lymphoma cells engineered to produce granulocyte-macrophage (GM)-CSF, and to a lesser extent cells producing IL-4, whereas vaccination with lymphoma cells transfected with the genes encoding IL-2 or B7-1 had no effect. The systemic immunity generated by GM-CSF- or IL-4-transfected lymphoma required both CD4⁺ and CD8⁺ T cells. Previous immunotherapeutic strategies for the treatment of lymphoma have focused on the generation of Ab responses targeted to the unique Ig Id as a tumor-specific Ag. Anti-idiotypic Abs were undetectable in animals vaccinated with GM-CSF-transduced lymphoma cells. In contrast, such immunization did result in the induction of Id-specific T cell responses. This is the first demonstration that T cell responses specific for a native tumor Ag are generated by GM-CSF-transduced tumor cell-based vaccination, suggesting that B cell lymphoma may be a suitable disease for genetically modified tumor vaccine strategies. *The Journal of Immunology,* **1996, 156: 3858–3865.**

Recent advances in the identification of factors regulating immune responses have led to a renewed interest in tumor vaccines. Because in most instances defined tumor Ags are not known, one general approach has been to utilize autologous tumor cells as a source of Ag. Such cell-based vaccine strategies seek to enhance the immunogenicity of the vaccinating tumor cells by modifying them in vitro to express immunomodulatory molecules such as cytokines or costimulatory molecules (1, 2). These studies have largely been conducted in murine nonhematopoietic tumor models, and have demonstrated the ability to generate systemic, tumor-specific immune responses capable of protecting mice against a subsequent tumor challenge or eradicating a small pre-established systemic tumor burden when administered as a therapeutic vaccine. Examination of the mechanism of tumor cell-based vaccine responses has demonstrated the requirement for tumor-specific T cell activation, as well as the critical role of augmented tumor Ag presentation, either by host APCs (3) or by the tumor itself (4). These preclinical studies form the basis for several ongoing clinical trials in the treatment of patients with solid tumor malignancies.

Unlike the solid tumors studied above, B cell lymphomas arise from cells with potent APC function. In many instances lymphoma lines can be shown to process and present Ag to T cells in vitro (5, 6). However, the ability of B cells to prime naive T cells in vivo is controversial, with some studies demonstrating failure to prime (7, 8), or even the induction of T cell tolerance (9, 10) upon encountering Ag on B cells. Consequently, the mechanisms whereby tumors of B cell origin evade T cell-mediated elimination may differ from epithelial tumors, as may the vaccine strategies that prove to be effective in treating them.

Because B cell lymphomas are clonal populations of transformed B cells, the Ig Id has been targeted as a "tumor-specific" Ag, uniquely expressed by the tumor cells (11). Attempts to generate an active host response to Id protein conjugated to carrier proteins have shown some success in both preclinical models as well as in small numbers of patients with low-grade B cell lymphomas. In these studies, production of anti-Id Abs by the host was associated with tumor regression (11–13). CD4⁺ T cells specific for Id protein have also been demonstrated, although in vitro evidence of cytolytic T lymphocyte (CTL)[3] activity (CD4⁺ or CD8⁺) has not been found with this approach (14).

Given what has been learned recently from the studies of genetically modified tumor cell-based vaccine strategies in nonlymphoid tumors outlined above, we sought to determine the efficacy

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[1] This work was supported by National Institutes of Health Grant P30 CA06973 and American Cancer Society Grant IM-737. H.I.L. is a recipient of National Institutes of Health K08 Clinical Investigator Award (CA01595).

[2] Address correspondence and reprint requests to Dr. Hyam Levitsky, Department of Oncology, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross Building, Room 364, Baltimore, MD 21205.

[3] Abbreviations used in this paper: CTL, cytolytic T lymphocyte; KLH, keyhole limpet hemocyanin; GM, granulocyte macrophage; HA, hemagglutinin; HRP, horseradish peroxidase.

0022-1767/96/$02.00

and immunologic mechanism of this approach in a model B cell lymphoma. Furthermore, the presence of a defined Ag, Ig Id, provides a unique opportunity for analysis of the response to a naturally occurring tumor-specific Ag in these systems.

Materials and Methods

Tumor cells

A20 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured in vitro in RPMI 1640 media, supplemented with 10% FCS, penicillin/streptomycin (50 U/ml), L-glutamine (2 mM), and β-mercaptoethanol (50 mM) (complete media), and grown as a suspension culture at 37°C, 5% CO_2. A20HAneo was selected and grown in complete media supplemented with the neomycin analogue G418 (400 μg/ml). A20IL-2 hygro, A20IL-4 hygro, and A20B7-1 hygro were selected in complete media supplemented with 400 μg/ml of hygromycin. A20HAB7-1 neo/hygro was created by transfection of A20HAneo after it was established as a stable cell line with the plasmid used to create A20B7-1 hygro, and selecting in both G418 and hygromycin. A20B7-1 hygro and A20HAB7-1 neo/hygro were further selected by FACS (Becton Dickinson Co., Mountain View, CA). Positive cells were sorted after staining with CTLA-4 Ig, the soluble fusion protein between the extracellular domain of CTLA-4 and human IgG γ-chain (15), and goat anti-human FITC (Cappel, West Chester, PA). All cell lines were periodically tested for, and found to be free of, mycoplasma contamination.

Gene transfer

Electroporation of A20 cells was used for plasmid transfection in the creation of A20-HA, A20-B7-1, A20-HAB7-1, A20-IL-2 (16), and A20-IL-4 (17). A20 cells (1 × 10^6) were resuspended in 1 ml of serum-free RPMI and mixed with 20 μg of linearized plasmid DNA. Cells were electroporated in a Bio-Rad Gene Pulser cuvette (0.4-cm electrode gap; Bio-Rad, Richmond, CA), shocked with 250 mV, 500 μF, and cultured in complete media for 24 h before drug selection. A20-GM-CSF was created by retroviral transduction using the retroviral construct MFG-mGMCSF as described (18).

ELISAs

Cytokine production by the tumor vaccines was quantified by plating 1 × 10^6 transfectants in 5 ml of complete media and collecting and filtering supernatant 24 h later. Mouse IL-2, IL-4, and granulocyte-macrophage (GM)-CSF were quantified using ELISA kits (Endogen, Boston, MA) according to the manufacturer's instructions.

T cell proliferation assay

Parental A20, A20HAneo, and A20HAB7-1 neo/hygro were γ-irradiated (5000 rad), and serial dilutions were performed in 96-well flat-bottom microtiter plates. T cells specific for influenza hemagglutinin (HA) were obtained from TCR-transgenic mice that carry the productively rearranged TCR α and β genes from a HA-reactive, I-E^d-restricted $CD4^+$ clone (19). This transgenic TCR clonotype is expressed on 15 to 20% of mature $CD4^+$ T cells. Transgenic mice were backcrossed onto a BALB/c background for more than 10 generations. Splenocytes were enriched for T cells by passage over nylon wool, and further depleted of MHC class II-bearing cells by magnetic depletion (MACS column) using mAb 14.4.4 (anti-I-E^d). The resulting cell population was <0.2% positive for I-E^d by FACS analysis and was shown to be unresponsive to Con A (5 μg/ml) in vitro, indicating adequate removal of splenic APCs. T cells (1 × 10^5 cells/well) were added to the irradiated A20 variants with or without the addition CTLA-4 Ig at a final concentration of 25 μg/ml, and were pulsed with [^{3}H]thymidine (1 μCi/well; Amersham, Arlington Heights, IL) after 3 days in culture. Cells were harvested 18 h later with a Packard Micromate cell harvester (Packard, Meriden, CT). Thymidine incorporation into DNA was measured as cpm on a Packard Matrix96 direct beta counter.

In vivo challenge protection assays

Six- to eight-week-old BALB/c female mice were used in all experiments, with 10 mice per group. All in vivo experiments were repeated at least once. Cells for injection were washed three times in HBSS. When indicated, vaccine cells were irradiated (5000 rad) and injected (0.1 ml) s.c. into the left flank using a TB syringe. Mice were challenged with live parental A20 injected i.v. through the tail vein. Mice were monitored twice weekly and killed after the development of tumor, which was evident as increasing abdominal girth and palpable abdominal mass.

In vivo Ab depletions

In vivo Ab depletions were started 1 wk before vaccination. mAb GK1.5 (20) was used for CD4 depletions and mAb 2.43 (21) was used for CD8 depletions. Ammonium sulfate-purified ascites fluid (titered at >1:2000 by staining of splenocytes on the FACS) was injected i.p. (0.1 ml per mouse) every other day for the first week and once per week afterward. Depletion of lymphocyte subsets was assessed on the day of vaccination, the day of live tumor challenge, and weekly thereafter by flow cytometric analysis of spleen cells stained with mAb 2.43 or GK1.5 followed by FITC-labeled goat Ab to rat IgG (Kirkegaard and Perry, Gaithersburg, MD). For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the other subsets.

Isolation of A20-derived Id protein

The A20 tumor cell line was adapted for sensitivity to hypoxanthine, aminopterin, and thymidine by growth in progressively increasing concentrations of 5-bromodeoxyuridine (Sigma Chemical Co., St. Louis, MO), as has been described by others (22). The resulting 5-bromodeoxyuridine-resistant A20 cells were then fused to the P3X63Ag8.653 nonsecreting, 8-azaguanine-resistant mutant myeloma cell line (CRL 1580; ATCC) with polyethyleneglycol (Sigma Chemical Co.) at a ratio of 1:4 (myeloma to A20). Specifically, the pelleted cell mixture was resuspended in 1 ml 40% polyethyleneglycol in PBS (calcium and magnesium free). After 1 min at 37°C, warm PBS was added slowly. The resuspended cells were pelleted again and resuspended in RPMI 1640/10% FCS and plated at 2 × 10^5/well in 96-well tissue culture plates. A 2× concentration of hypoxanthine, aminopterin, and thymidine medium was added in a volume of 0.1 ml to each well 24 h later. Supernatants of secreting hybrids were screened in an ELISA, in which intact Ig was captured with goat anti-mouse IgG2a isotype-specific Abs coating microtiter plates and by detection with horseradish peroxidase (HRP)-goat anti-mouse κ-Abs (Caltag, South San Francisco, CA). Hybrids were not cloned.

One hybrid cell line was expanded for growth in vitro, and collected supernatants were passed over a protein G-Sepharose column (Pharmacia, Piscataway, NJ). Elution of bound A20 IgG2a (Id) was performed according to the manufacturer's recommendations. Id isolated in this way was dialyzed against PBS before further use. SDS-PAGE analysis documented an estimated purity of greater than 90%.

Formal demonstration that the Id was tumor derived was provided by the demonstration that serum from mice immunized with 50 μg of A20 Id chemically conjugated to keyhole limpet hemocyanin (KLH) specifically bound A20 tumor cells by flow cytometry analysis (data not shown).

ELISA for serum anti-idiotypic Ab

Serum was diluted over microtiter plates coated with Id (10 μg/ml). Bound Ab was detected with HRP-goat anti-mouse IgG1 isotype-specific Abs (Caltag). For each experiment, serum samples were obtained from five randomly selected mice per group and analyzed individually. Results with HRP-conjugated goat anti-mouse IgM (μ-chain specific), IgG2b, and IgG3 Abs (Caltag) were uniformly negative.

Id-specific T cell response

Immune T lymphocytes were obtained from spleens after passing over a nylon wool column and were placed in 96-well flat-bottom microtiter plates (200 μl, 2 × 10^5 cells/well) along with Id at a concentration of 100 μg/ml. Irradiated (2000 rad) spleen cells from normal BALB/c mice were also added to these cultures (5 × 10^5 cells/well) as a source of APCs. Cultures were maintained at 37°C, 5% CO_2 for 7 days. Supernatants were then harvested, pooled within each group, and subjected to analysis of IFN-γ (Life Technologies, Inc., Gaithersburg, MD) or IL-4 levels by ELISA (Endogen).

Results

Establishment and characterization of the model system

We sought to establish a murine lymphoma model system that satisfies several criteria with respect to potentially relevant immunologic parameters. These include having the cell surface Ag profile of a B cell lymphoma, expressing a monoclonal membrane-bound Ig, having normal levels of MHC class I and class II Ags, having inducible expression of T cell costimulatory molecules, and having the ability to process and present exogenous Ags to $CD4^+$ T cells. In addition, its behavior in vivo should approximate some

forms of lymphoma seen clinically with regard to sites of disease and tumor trafficking. In all of these respects, the BALB/c lymphoma A20 was found to be a suitable model. This spontaneously arising tumor has been extensively studied in vitro as a model for the analysis of Ag processing and presentation (23). Molecular characterization of A20 shows it to have features of a mature B cell, having undergone Ig heavy and light chain gene rearrangement and heavy chain class switching. Consequently, the tumor expresses a monoclonal surface IgG2a. Flow cytometric analysis demonstrates the expression of normal levels of MHC class I and high levels of MHC class II (Fig. 1*A*). Staining of unstimulated parental A20 for expression of the B7 family costimulatory molecules using the chimeric counter-receptor CTLA-4 Ig fails to detect significant levels of B7 expression by FACS analysis, although expression is induced after activation with LPS (Fig. 1*C*). In spite of the constitutively low levels of B7-1 and B7-2 present on resting A20 cells, functional evidence of B7 mediated costimulation is seen with CTLA-4 Ig blocking of Ag-specific T cell activation when mixed with Ag-expressing A20 cells in vitro (Fig. 2), suggesting either that the low levels detected are adequate for delivering the costimulatory signal or that T cell interaction with A20 results in B7 up-regulation during the course of the assay. Taken together, these features in part account for the ability of processed exogenous or membrane Ags to be effectively presented to MHC class II-restricted T cells.

When injected i.v., the tumor traffics to lymphoid tissues, with infiltration of the mesenteric lymph nodes and the formation of splenic tumor nodules, as well as nodules on the surface of the liver. Tumor can also be found infiltrating the bone marrow and circulating in the peripheral blood (data not shown). Injection of as few as 1×10^3 A20 cells i.v. will result in progression to a lethal tumor burden in a fraction of mice, with 1×10^4 cells being the minimal LD_{100}. The tumor is relatively nonimmunogenic, in that immunization with irradiated A20 cells affords little protection against a subsequent live tumor challenge.

Creation of genetically modified A20 tumor vaccine

We sought to create and test genetically modified A20 tumor vaccines engineered to produce cytokines or costimulatory molecules that have been found to enhance tumor immunogenicity in other, nonhematopoietic tumor systems. Stable transfectants of A20 were created using constructs encoding for IL-2, IL-4, GM-CSF, and B7-1. The cytokine gene-transfected A20 lines produced levels of cytokine comparable to production rates reported in other active tumor vaccine models (A20-IL-2: 60 U/10^6 cells/24 h; A20-IL-4: 12 ng/10^6 cells/24 h; and A20-GM-CSF: 130 ng/10^6 cells/24 h). The resulting lines were similar to parental A20 with respect to MHC class I, class II, or B7 expression (data not shown). The transfected A20-B7-1 cell line expressed B7 levels significantly elevated above the parental baseline, even after LPS activation (Fig. 1*C*). In fact, the FACS profile of B7 expression by A20-B7-1 is comparable to that seen on mature dendritic cells (Fig. 1*B*). In vitro evidence for T cell recognition of the enhanced B7-1 expression achieved by transfection is seen in Figure 2 where, on a cell for cell basis, the B7-1 transfectant is 10-fold more potent an APC than A20 expressing endogenous levels of B7.

Comparison of A20 vaccines in a challenge protection model

Early studies of genetically modified tumor vaccines focused on the local rejection of live vaccine cells. It has been subsequently appreciated that the character of the local response is often quite

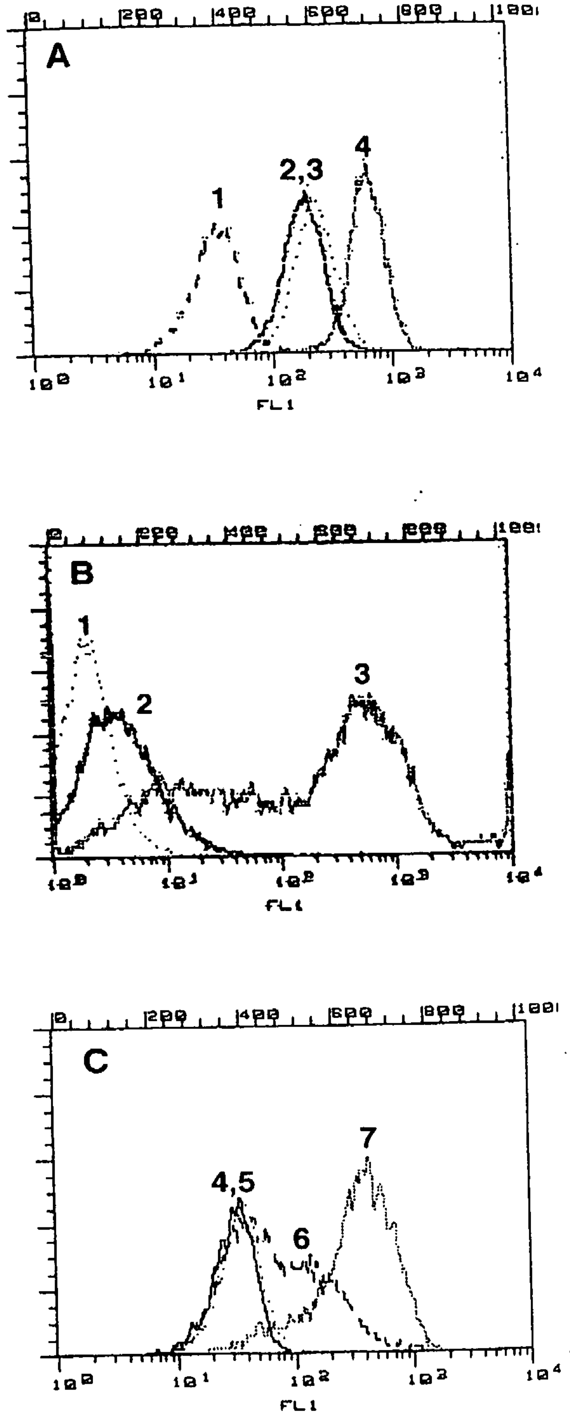

FIGURE 1. *A*, Expression of MHC class I and II Ags on parental A20. A20 cells were stained with: 1) OKT3 primary (negative control), 2) 34-4-20s primary (anti-D^d), 3) 34-1-2s primary (anti K^d), or 4) 14.4.4 primary (anti I-E^d). Secondary stain was goat anti-mouse IgG2a FITC for all samples. *B*, Expression of B7 on mouse B cells and dendritic cells, compared with *C*, A20 variants. Groups are stained with CTLA-4 Ig (chimeric ligand for B7) primary and goat anti-human IgG FITC secondary, or secondary Ab alone. 1) Resting B cells; 2) LPS-activated B cells (5 μg/ml × 48 h); 3) bone marrow-derived dendritic cells cultured in GM-CSF × 7 days; 4) parental A20; 5) A20 secondary Ab only; 6) LPS-activated A20; and 7) A20-B7-1.

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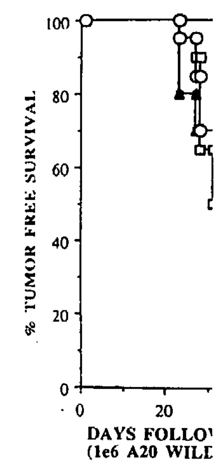

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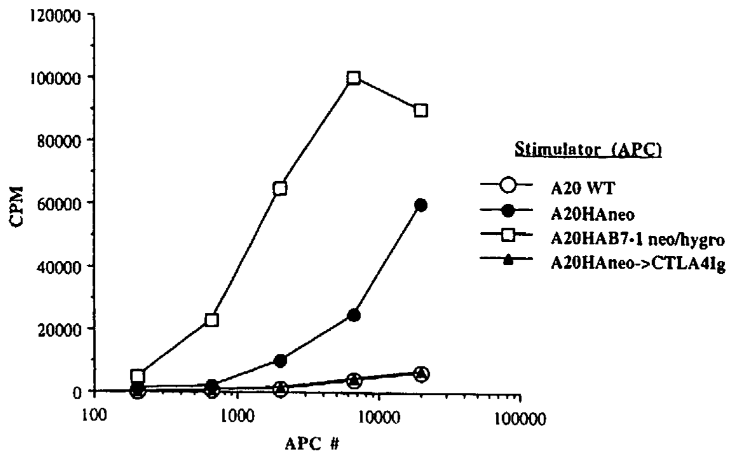


FIGURE 2. Effect of enhanced B7-1 expression on the magnitude of the T cell proliferative response. The gene for influenza HA was transfected into A20, and stable lines were selected to create A20HAneo. This line was subsequently transfected with the gene for B7-1 and sorted by FACS for high B7-1 expression, creating A20HA-B7-1 neo/hygro. The lines were irradiated (5000 rad), and serial dilutions were plated, followed by the addition of anti-HA T cell receptor-transgenic T cells with or without the addition of CTLA-4 Ig. [^{3}H]thymidine incorporation was determined at 3 days. The experiment was performed twice with similar results.

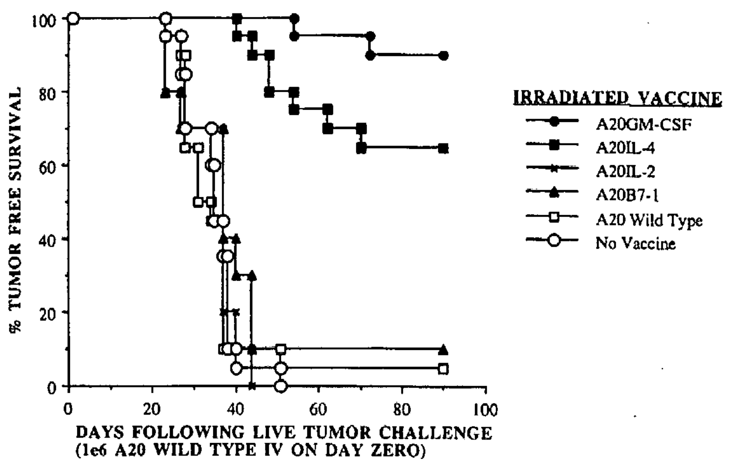


FIGURE 3. Protection against a systemic challenge of A20 lymphoma using modified tumor cell vaccines. Mice were immunized with 1×10^6 irradiated A20 variants s.c., and challenged i.v. 2 wk later with 1×10^6 live parental A20 cells. Inspection for tumor occurred twice weekly and animals were killed upon developing clear evidence of progressive lymphoma (increasing abdominal girth and palpable abdominal mass). Shown is a Kaplan Meier plot of tumor-free survival, pooling the results of two separate experiments, with 10 mice per group for each experiment.

distinct from the response mediating systemic immunity, and consequently the capacity to reject live vaccine cells does not necessarily predict the ability to reject a parental tumor challenge at a distant site. Of the four constructs described above, only A20-IL-4 is consistently rejected when 1×10^6 live cells are injected s.c. The rate of tumor outgrowth is slowed in the case of A20-IL-2 and A20-GM-CSF compared with parental A20, and a fraction of mice do reject the vaccine. A20-B7-1 grows out with identical kinetics to A20 wild type (data not shown). To compare the ability to generate a systemic antitumor immune response, the vaccines were irradiated and injected s.c., followed 2 wk later by a live i.v. challenge with 10^6 parental A20 cells (Fig. 3). Mice immunized with irradiated A20-GM-CSF were protected from developing systemic lymphoma following live tumor challenge. Irradiated A20-IL-4 induced a lesser, but still significant, degree of protection. In contrast, animals immunized with irradiated A20-IL-2, A20-B7-1, and A20 wild type developed evidence of systemic lymphoma at a rate comparable to unimmunized controls. Interestingly, no systemic immunity was generated when any of the A20 vaccine constructs were injected i.v. (data not shown).

Analysis of the mechanism of the response to A20-GM-CSF and A20-IL-4

We wished to determine the relative roles of the cellular and humoral immune responses in the systemic immunity generated by A20-GM-CSF and A20-IL-4. Mice were depleted of CD4$^+$ or CD8$^+$ T cells before immunization with A20-GM-CSF or A20-IL-4. Depletion was maintained for the duration of the experiment. Two weeks following immunization, the mice were challenged i.v. with live parental A20 as before. The presence of both CD4$^+$ and CD8$^+$ T cells was found to be critical to the generation of systemic immunity to A20 with these cell-based vaccines, as depletion resulted in a profound reduction in tumor-free survival (Fig. 4).

Given the association between tumor rejection and anti-Id Ab titer in Id-based vaccine systems, we examined the anti-Id humoral response in mice immunized with irradiated A20-GM-CSF, A20-IL-4, or parental A20, and compared these to the response generated by A20 Id protein conjugated to KLH (Fig. 5). Although a high titer of anti-Id Ab is raised in the A20 Id protein conjugated to KLH-primed mice, immunization with the cell-based vaccines resulted in no detectable titers of anti-Id Ab. It thus seems unlikely that

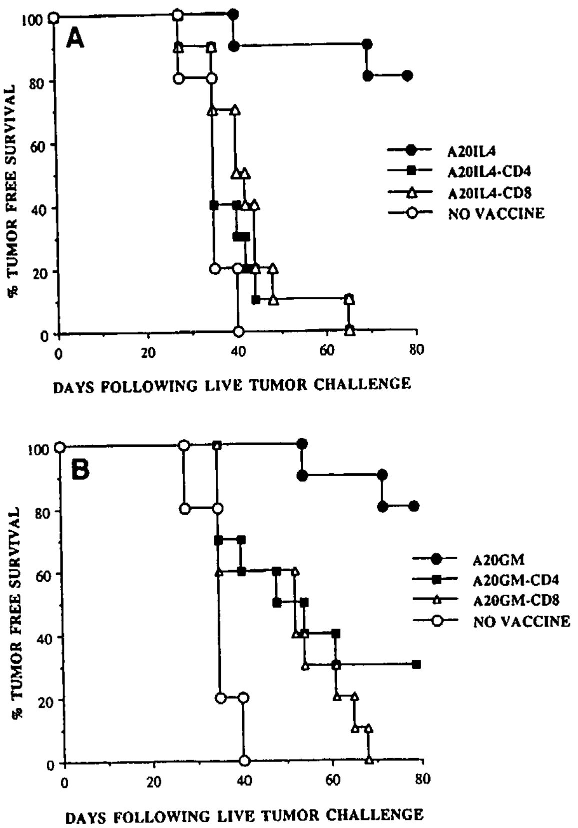


FIGURE 4. The role of CD4$^+$ and CD8$^+$ T cells in the generation of systemic immunity by A20-IL-4 and A20-GM-CSF vaccines. Mice were depleted of either CD4$^+$ or CD8$^+$ T cells by injection of mAb beginning 1 wk before immunization and continuing for the duration of the experiment. Mice were immunized with *A*, A20-IL-4 or *B*, A20-GM-CSF s.c. and challenged 2 wk later i.v. with 1×10^6 parental A20 cells.

this Ab response to Id plays a direct role in the A20-GM-CSF- or A20-IL-4-primed response against a systemic A20 tumor challenge.

The presence of Id protein uniquely expressed by the tumor allows for the measurement of T cell responses to a candidate tumor-specific Ag. Mice immunized s.c. with irradiated parental A20 or A20-GM-CSF were killed 2 wk after priming, and splenocytes were tested in vitro for recognition of purified A20 Id (Fig. 6). A20 Id-specific IL-4 release was seen from A20-GM-CSF-primed mice, but not from irradiated parental A20-primed controls. In contrast, while some IFN-γ was released in response to Id in vitro, the levels seen from A20-GM-CSF-primed splenocytes were not significantly greater than those seen when irradiated wild-type A20 was used as vaccine.

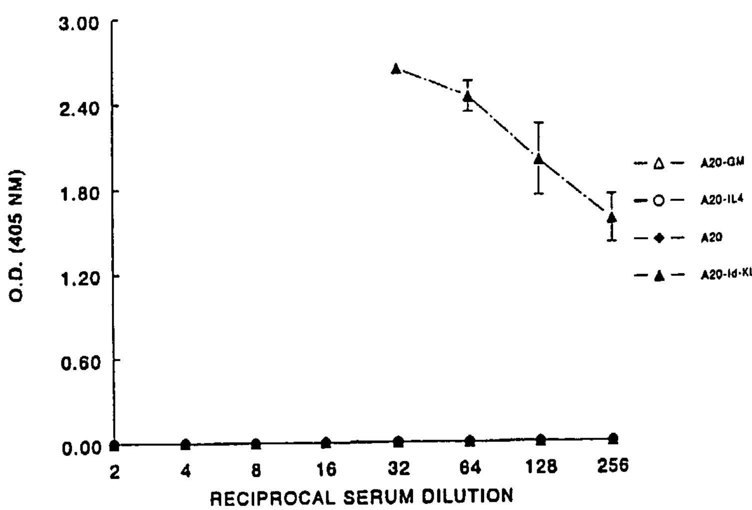
Immunization with A20-GM-CSF and A20-IL-4 can eradicate pre-established systemic lymphoma

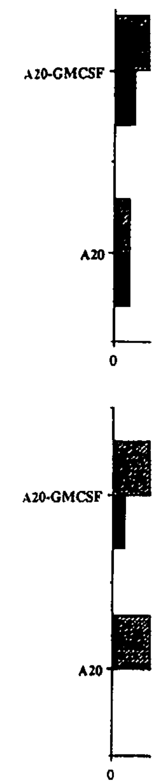
A potentially relevant feature of the A20 model is the fact that tumor traffics to lymph node and spleen, two sites critical to the generation of Ag-specific immune responses. It is therefore important to determine whether animals with a pre-established tumor burden can be treated in a "therapy" model of disease. Mice were injected i.v. with 10^5 A20 cells on day 0 and subsequently treated with irradiated A20-GM-CSF, A20-IL-4, or a 1:1 mixture of the two on day 5 (Fig. 7). Significant protection against progression to bulky disease was achieved with both A20-GM-CSF and A20-IL-4. The experiment was terminated 100 days after the initial tumor challenge, and autopsy of surviving mice failed to reveal evidence of occult lymphoma. There did not appear to be an additive or synergistic effect between the two vaccines at the doses used. The ability to successfully immunize mice with a small pre-established tumor burden at a time when lymphoma can be demonstrated in the lymph nodes and spleen suggests that the integrity of the immune response is not significantly disrupted in the early stages of disease.

Discussion

These studies demonstrate the ability to generate a systemic antitumor immune response to a B cell lymphoma by immunization with GM-CSF- or IL-4-producing autologous tumor vaccines. Immunization with these constructs results in the ability to eradicate a pre-established tumor burden that progresses to widely disseminated lymphoma in untreated animals. The paracrine production of these cytokines by tumor cells has been shown to result in the generation of effective immune responses in certain nonhematopoietic tumors as well (17, 18, 24). In these systems, evidence

FIGURE 5. Analysis of the anti-idiotype Ab response generated by irradiated parental A20, A20-IL-4, and A20-GM-CSF cells, vs A20-idiotype conjugated to KLH. Mice were immunized s.c. with 1×10^6 irradiated vaccine cells or 25 μg of A20-Id. Two weeks later, serum was collected and ELISA performed as described. Shown are the mean values ± SE of five individual mice per group.





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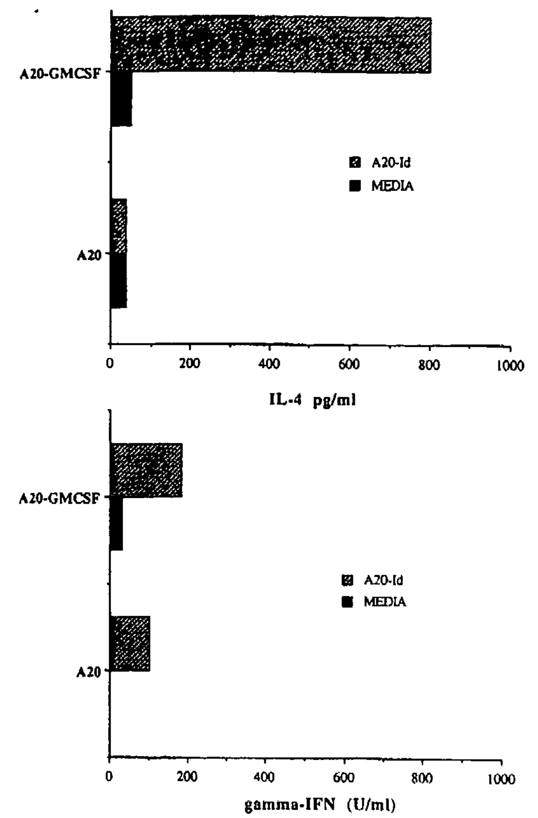
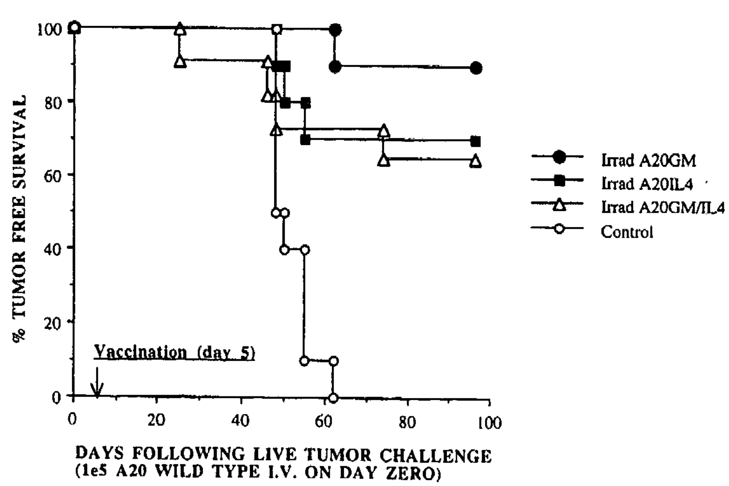


IGURE 6. Idiotype-specific T cell responses generated by vaccination vith A20-GM-CSF. Mice were immunized with 1×10^6 irradiated paental A20 or A20-GM-CSF cells. Spleens were harvested on day 9, and iylon wool-enriched T cells were added to naive spleen cells as a source f APCs. Supernatants were collected from wells pulsed with A20 idioype or media control after 7 days in culture. ELISA measurements for IL-4 nd IFN-γ were performed as described in *Materials and Methods.*

upports a mechanism whereby GM-CSF and IL-4 recruit and acivate host APCs that internalize, process, and present tumor Ag to cells. In contrast, immunization with lymphoma cells engineered to produce IL-2 or express elevated levels of B7-1 failed to generate significant systemic immunity. These vaccine strategies have been shown to prime systemic antitumor immunity in certain nonhematopoietic tumor systems (16, 25–28). The inferred mechanism has been that IL-2 production or enhanced B7-1 expression by the tumor provides a costimulatory or "second signal" to responding T cells, enabling them to be directly primed upon encountering Ag presented on the tumor's MHC molecules. B7-1 expression has been shown to further enhance the priming of systemic antitumor immunity in a murine sarcoma model when cotransfected with the genes encoding MHC class II molecules, resulting in $CD4^+$ T cell-mediated tumor rejection (28). Given that B cell lymphomas represent MHC class II-positive neoplasms derived from cells capable of some degree of Ag-specific T cell activation, it is perhaps surprising that strategies aimed at enhancing direct Ag presentation by the tumor are ineffective in this tumor system. The costimulatory capacity of human lymphoma cells was examined by Schultze et al. (29). Follicular lymphoma cells obtained from seven patients failed to stimulate significant allogeneic T cell proliferative responses. These tumors expressed normal levels of MHC class I and II Ags, but very low levels of the costimulatory molecules B7-1, B7-2, and B7-3. In vitro activation of the lymphoma cells by culturing them in the presence of cells transfected with CD40 ligand resulted in up-regulation of intercellular adhesion molecule-1, LFA-3, and the B7 family members. Lymphoma cells activated in this fashion subsequently induced strong allogeneic T cell responses that were blockable by CTLA-4 Ig. These findings are similar to our results characterizing the effect of B7 expression of A20 on the potency of its APC function. Nevertheless, our results demonstrated that achieving a level of B7-1 expression that dramatically enhances APC function in vitro was inadequate to prime a sufficient antitumor response in vivo.

One explanation for these findings may relate to the relative lack of immunogenicity of A20, since it has been shown that the systemic immunity induced by B7-1-transfected tumor cells appears to correlate with the inherent immunogenicity of the tumor (30). In fact, immunization with the relatively nonimmunogenic B16F10 melanoma transfected with B7-1 results in the rejection of the live transfectant, but fails to prime systemic immunity to the parental tumor (31). Further analysis of the mechanism of priming by the B7-1-transfected tumor has formally demonstrated that although MHC class I-restricted CTL can be directly primed by the B7-1-transfected tumor, this mechanism appears to be far less efficient



IGURE 7. Treatment of established disseminated 20 lymphoma with irradiated A20-GM-CSF vs 20-IL-4. Mice were injected i.v. with 1×10^5 paental A20 cells on day 0 and vaccinated s.c. with $\times 10^6$ irradiated A20-GM-CSF, A20-IL-4, or a mixure of 1.5×10^6 cells of each on day 5. Animals ere followed twice weekly for the development of imor.

than "cross-priming" of CTL by host APCs that have taken up exogenous tumor Ags (4). Presumably, the efficacy of latter pathway in mediating systemic antitumor immunity is critically dependent on the existence of a strong tumor rejection Ag.

Another factor that may impact on the efficacy of "tumor as APC" in the priming of CD4$^+$ T cells relates to the pathways of Ag trafficking. Antigenic peptides presented on MHC class II of the lymphoma are either derived from membrane proteins (such as the model Ag HA used in this study) or exogenous proteins that are internalized through receptor-mediated endocytosis or fluid phase pinocytosis. If the relevant tumor Ags are derived from nuclear or cytoplasmic proteins, endogenous invariant chain would prevent these from gaining access to the MHC class II compartment. In fact, in the MHC class II-transfected fibrosarcoma model above, cotransfection with invariant chain abrogated the antitumor immune response (32). Finally, it is possible that enhanced B7-1 expression alone is insufficient for priming naive T cells in vivo, as some studies have suggested that even activated B cells, which have been shown to express increased levels of B7-1 and B7-2 (33, 34), induce tolerance in vivo (9, 10).

The systemic immunity generated by A20-GM-CSF and A20-IL-4 requires the participation of CD4$^+$ and CD8$^+$ T cells. In contrast, conjugated Id vaccines have been characterized as predominantly inducing an anti-Id humoral response that is cytotoxic in vitro and can mediate systemic responses in passive transfer experiments (11, 12). A20-IL-4 and A20-GM-CSF failed to raise a significant titer of anti-Id Ab, making a role for direct humoral-mediated antitumor immunity unlikely. In spite of the absence of Ab production, we found clear evidence for Id-specific T cell responses to A20-GM-CSF immunization. The potential utility of generating Id-specific T cell responses in the immunotherapy of B cell lymphoma is supported by studies demonstrating that B cells (and their transformed counterparts) can process and present their Ig Id for recognition by MHC class II-restricted CD4$^+$ T cells (35). In vitro evidence of CD4$^+$ and CD8$^+$ T cell priming against Id has been reported in an immunogenic B cell hybridoma model using irradiated tumor cells as vaccine (36, 37). In our system, the generation of an Id-specific T cell response required paracrine production of GM-CSF by the tumor cells, as vaccination with irradiated parental A20 cells failed to generate this response. While A20-GM-CSF vaccination resulted in both an anti-Id T cell response and systemic immunity to the lymphoma, it is unclear what role this Ag-specific response plays in the rejection of A20. This question is currently under study. It is particularly interesting that IL-4 is the dominant cytokine released from lymphocytes primed with the A20-GM-CSF vaccine and pulsed with A20 Id in vitro, suggesting a Th2 pattern. This is consistent with several features of the response to GM-CSF-producing tumor vaccines seen in solid tumor systems, including the presence of IL-4 mRNA in the lymph nodes draining the vaccine site, the induction of an eosinophilic infiltrate, and loss of vaccine-induced protection with the coadministration of systemic IFN-γ (K. Hung et al., manuscript in preparation).

The strategies explored in this study suggest that B cell lymphoma is a suitable disease for consideration of cell-based vaccine therapies. As one of our goals of ongoing lymphoma vaccine development is the induction of potent cell-mediated immune responses (38), such results may provide a rationale for a clinical strategy of priming with an autologous cellular vaccine, followed by boosting with Id protein. The ability to obtain large numbers of tumor cells at diagnosis, achieve minimal residual disease with chemotherapy, and have a sensitive marker of relapse and response should facilitate the translation of these findings to clinical trials.

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